AF IAW

PATENT
Attorney Docket No. P2133US
Client Ref. No. DM-3341; JHU 101.1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Levitsky et al.

Art Unit: 1632

Application No. 09/992,443

Examiner: Li, Q.J.

Filed: November 16, 2001

For: A UNIVERSAL IMMUNOMODULATORY CYTOKINE-EXPRESSING BYSTANDER CELL LINE AND RELATED COMPOSTIONS AND METHODS OF MANUFACTURE AND USE

TRANSMITTAL OF APPELLANTS' REPLY BRIEF

Mail Stop Appeal Brief – Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

In accordance with 37 CFR 1.192, Appellants hereby submit Appellants' Reply Brief in triplicate.

The items checked below are appropriate:

1. Status of Appellant

This application is on behalf of ☐ other than a small entity or ☒ a small entity.

2. Oral Hearing

☐ Appellant requests an oral hearing in accordance with 37 CFR 1.194.
☐ Appellant requested an oral hearing in accordance with 37 CFR 1.194 at the time appellant filed Appellant's Brief on Appeal.

3. Extension of Time

☐ Appellant petitions for a one-month extension of time under 37 CFR 1.136, the fee for which is $110.00.

☒ Appellants believe that no extension of time is required. However, this conditional petition is being made to provide for the possibility that appellants have inadvertently overlooked the need for a petition and fee for extension of time.

Extension fee due with this request: $

4. Total Fee Due

The total fee due is:

Request for Oral Hearing	$ 0.00
Extension Fee (if any)	$110.00.

Total Fee Due: $

5. Fee Payment

☐ Attached is a check in the sum of $.
☐ Charge Account No. 07-0181 in the sum of $. A duplicate of this transmittal is attached.

6. Fee Deficiency

☒ If any additional fee is required in connection with this communication, charge Account No. 07-0181. A duplicate copy of this transmittal is attached.

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Date: January 26, 2005

CH02/ 22364889.1

PATENT
Attorney Docket No. P2133US
Client Ref. No. DM-3341; JHU 101.1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of:

Levitsky et al. Art Unit: 1632

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Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

APPELLANTS' REPLY BRIEF

Sir:

The Appellants submit this Reply Brief in response to the Examiner's Answer mailed on November 26, 2004.

As an initial matter, on page 6 of the Examiner's Answer, the Examiner agrees that the term "naturally" as used in the specification is opposite to "modified" (by the hands of man). The Examiner concludes that "naturally lacks MHC-I and MHC-II antigens" encompasses cell lines, which never had MHC-I and MHC-II antigens, and cell lines, which lost MHC-I and MHC-II antigens due to a cancerous mutation.

However, on pages 7 and 8 of the Examiner's Answer, in the guise of a written description rejection, the Examiner states that the specification fails to describe an RBC or any other human cell line that "naturally lacks MHC-I and MHC-II antigens" and fails to describe an actual reduction to practice of a human cell line that naturally lacks MHC-I and MHC-II antigens, but only describes K562 cells. The Examiner takes the position that K562 is the only cell line disclosed by Appellants within the specification or in subsequently

submitted references that naturally lacks MHC-I and MHC-II antigens and that, therefore, Appellants fail to provide representative species for the claimed genus, i.e., human cell lines that never had or lost MHC-I and MHC-II antigens. This is inconsistent with the definition of "naturally" accepted by the Examiner on page 6 of the Examiner's Answer.

Throughout the prosecution of this case, the Examiner has been concerned with the meaning of "naturally lacks." Appellants submit that, given the acknowledgment by the Examiner that naturally lacks means "never had or lost," it is irrelevant as to how the cell line became negative for MHC-I and MHC-II antigens (e.g., whether due to a "cancerous mutation" or something else).

Appellants submit that the specification not only provides K562 as an example of a cell line that naturally lacks (never had or lost) MHC-I and MHC-II antigens, the specification teaches one of ordinary skill in the art how to identify and characterize other such cell lines using the primary monoclonal antibodies W632 (anti-human class I heavy chain) and L243 (anti-human class II). Thus, contrary to the position taken by the Examiner on page 12 of the Examiner's Answer, Appellants submit that the specification describes a cell line (K562) that naturally lacks (never had or lost) MHC-I and MHC-II antigens and enables one of ordinary skill in the art how to identify other such cell lines.

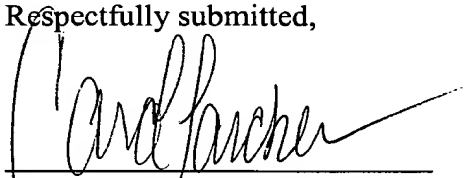
On page 12, the Examiner also argues that the K562 cell line was derived 30 years ago and similar lines have not been described. Appellants respectfully disagree. For example, Sullivan et al., J Clin Invest. 1985 Jul; 76(1): 75-9 (copy enclosed), describe analysis of lymphocytes from a patient with bare lymphocyte syndrome. Class I and class II HLA antigens are not normally expressed on the surface of such lymphocytes, suggesting a pretranslational regulatory defect of both class I and β2-microglobulin gene expression.

With respect to the Examiner's comments regarding Wang et al. (J. Clin. Invest., 1993, copy enclosed) and the expression of MHC I and II by the SK-MEL-33 cell line, the Examiner relies on Winchester et al. (PNAS, 1978, copy enclosed) for the proposition that a number of melanoma lines, including SK-MEL-33 cells, are 100% positive for expression of the MHC-II antigen, Ia. Appellants respectively submit that, not only does Winchester et al. state that the cells were analyzed for an "Ia-like" antigen, which they call "Ia" even though it is described as "distinct from classic HLA molecules" (page 6235, first column), Table 1 of Winchester et al. shows that the results of staining SK-MEL-33 cells for the "Ia-like" antigen

were "tr-3," indicating that only a trace amount of the antigen was detected. The Examiner can hardly rely on these data to state that SK-MEL-33 cells are HLA Class II-positive.

Appellants submit that the specification provides an adequate description of an exemplary cell line that naturally lacks (never had or lost) major histocompatibility class I (MHC-I) antigens and major histocompatibility class II (MHC-II) antigens and enables one of ordinary skill in the art how to identify and characterize other such cell lines.

Respectfully submitted,



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Date: January 26, 2005

CERTIFICATE OF MAILING

I hereby certify that the attached APPELLANTS' REPLY BRIEF (along with any documents referred to as being attached or enclosed) is being deposited with the United States Postal Service on the date shown below with sufficient postage as first class mail in an envelope addressed to: Mail Stop Appeal Brief – Patents, Commissioner of Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

Date: January 26, 2005

Angelica Diaz

Angelica Diaz

CH02/ 22364839.1

Molecular Analysis of the Bare Lymphocyte Syndrome

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Abstract

The bare lymphocyte syndrome is a disorder in which class I histocompatibility antigens fail to be expressed normally on the surface of lymphocytes. Utilizing complementary DNA probes for both β_2-microglobulin and class I genes, the molecular basis for this syndrome was investigated in a family with two siblings exhibiting the bare lymphocyte syndrome. Southern blot analysis demonstrated no gross internal defect in either class I or β_2-microglobulin genes. Northern blot analysis of class I and β_2-microglobulin messenger RNAs also revealed no qualitative difference between affected and unaffected family members. In contrast, quantitation of both class I and β_2-microglobulin transcripts demonstrated each to be decreased in patients when compared to controls. Moreover, the decrease in both transcripts was coordinate. These results suggest that the bare lymphocyte syndrome may represent a pretranslational regulatory defect of both class I and β_2-microglobulin gene expression.

Introduction

The bare lymphocyte syndrome is defined as a syndrome in which a patient's lymphocytes cannot be tissue-typed by standard serologic cytotoxicity tests. Touraine et al. (1) described a child presenting with severe combined immunodeficiency in the initial case report of 1978. Since then, several forms of the syndrome have been identified. A defect in class I major histocompatibility antigen expression has been described with and without concomitant severe combined immunodeficiency (2–7). Several case reports of a class II major histocompatibility defect have appeared recently. This class II defect may be isolated (8) or associated with a defect in class I expression (9–11) and has a variable phenotype. We have previously described two siblings without immunodeficiency who exhibit the bare lymphocyte syndrome. The older affected child presented in 1980 with aplastic anemia but had normal IgA, IgG, and IgM levels. The younger affected child has been healthy. Neither has suffered from any opportunistic infections nor an unusual number of viral infections (7). Analysis of these patients may help clarify the factors involved in the regulation of cell surface expression of class I antigens and may contribute to our understanding of class I antigen functions. Here, for the first time is a demonstration of a pretranslational defect in both class I and β_2-microglobulin gene expression associated with the bare lymphocyte syndrome.

This work was presented in part at the American Association of Physicians meeting, Washington, DC, in May 1984.

Ms. Sullivan is a Medical Scientist Training Program student at the University of California, San Francisco Medical Center; Dr. Peterlin is an assistant and Dr. Stobo is an investigator with the Howard Hughes Medical Institute.

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J. Clin. Invest.

0021-9738/85/07/0075/05 $1.00
Volume 76, July 1985, 75–79

Methods

Cells. Family "L," previously described (7), was used as the sole source of "bare lymphocytes" for the experiments described. An affected child (AC2; see Fig. 1) was identified during routine tissue typing in consideration for bone marrow transplant as treatment for aplastic anemia. At that time, another affected child (AC4) was also identified as having the bare lymphocyte syndrome. Fresh peripheral blood mononuclear cells (PBMCs),[1] consisting of lymphocytes and monocytes, were isolated through a Ficoll-Hypaque gradient. Fresh circulating leukocytes (lymphocytes, monocytes, and granulocytes) were isolated in 1.5% dextran (200,000 mol wt). Epstein-Barr virus (EBV)-transformed lymphocytes from the family members were continuously maintained in RPMI 1640 supplemented with 10% fetal calf serum and the usual antibiotics.

Northern and RNA dot blot analysis. Three probes were utilized in these analyses. The β_2-microglobulin probe (gift of K. Itakura) is a 550-base-pair (bp) complementary (c) DNA clone (12). The class I probe (gift of S. Weissman) is homologous to HLA B7 but cross-hybridizes to HLA-A, -B, and -C genes (13). The actin probe is a 1.5-kilobase (kb) cDNA clone (gift of P. Gunning) (14). The gift of ribosomal cDNA was generated from L cells by Andree Dozy. RNA was isolated by the guanidinium method (15). For the Northern blot in Fig. 2-C, 25 μg of total RNA was isolated from the EBV-transformed lines, denatured in formaldehyde as previously described (16, 17), and applied to a 0.8% agarose gel. For the Northern blot in Fig. 5, 5 μg of total RNA isolated from PBMCs was used. The gel was photographed using a silica thin-layer chromatography plate to identify the positions of the 18S and 28S bands. Blots were baked and incubated with a 50% formamide solution at 42°C, as previously described (17). 50 ng of probe, nick-translated to a specific activity of 10^8 cpm/μg was denatured by boiling and added to 10 ml of fresh formamide solution. After 24 h of incubation the blots were washed in 0.1 × standard saline citrate (pH 7.0), 0.1% sodium dodecyl sulfate (SDS) at 55°C as previously described (17). Northern dot blots utilized total RNA isolated as above from leukocytes. For the dot blots, 1 μg of RNA was denatured as above. Twofold dilutions of RNA were made into microtiter wells with 20-strength SSC. The RNA was then applied to nitrocellulose and probed as above.

Fluorescent analysis. Three antibodies from Becton-Dickinson & Co. (Mountain View, CA) were used in the identification of subpopulations of PBMC. Leu 1 (clone L17F12) depicts most T cells (18). HLA-D-related (DR) (clone L243)-specific antibody binds nonpolymorphic regions of class II molecules (19). The M3 (clone Mϕ P9) antibody is specific for 70–90% of mature forms of circulating monocytes (20). These three antibodies are linked to phycoerythrin, which emits at a wavelength distinct from that of fluorescein isothiocyanate (FITC) and allows simultaneous two-color analysis (21). Two other antibodies were used to analyze the display of class I antigens on the surface of PBMC. Anti-β_2-microglobulin (clone L368) linked to FITC was purchased from Becton-Dickinson & Co. (19). W6/32 is a monoclonal antibody that recognizes a nonpolymorphic determinant on class I

1. *Abbreviations used in this paper:* EBV, Epstein-Barr virus; FITC, fluorescein isothiocyanate; PBMC, peripheral blood mononuclear cell(s).

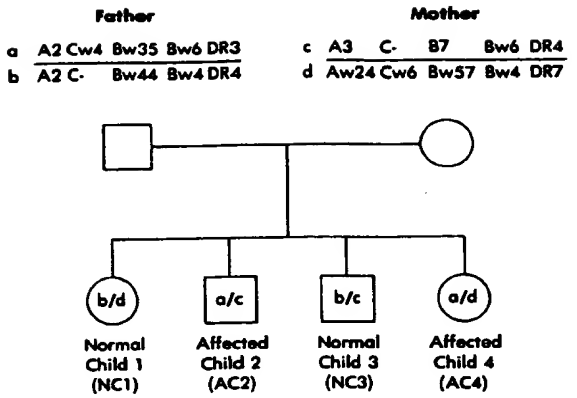

Figure 1. Pedigree. Family "L" consists of the unaffected mother (*M*) and father (*F*), two unaffected children (*NC1, NC3*), and two affected children (*AC2, AC4*).

molecules (22). Fluorescent analysis using W6/32 was performed using an indirect immunofluorescent technique. Flow cytometry was performed on a FACS IV (Becton-Dickinson & Co.) as previously described. (21, 23).

Results

To examine the structure of the class I and the β_2-microglobulin genes, Southern blots were prepared from DNA isolated from the family members' PBMCs. Southern blot analysis demonstrated no gross internal rearrangements or deletions in either the class I genes or the β_2-microglobulin genes of the affected children when compared to the normal siblings or the parents (data not shown and reference 7). In an effort to analyze the transcripts from these genes, RNA was isolated from the EBV-transformed cells of the family. Six weeks after EBV transformation, "bare lymphocytes" have been found to have normal class I surface expression (5, 7). Hybridization of Northern blots with the β_2-microglobulin probe revealed bands ~1 kb in length for all family members (Fig. 2). There was no qualitative difference between the affected and the unaffected family members. When the Northern blots were hybridized with the B7 probe, specific for class I antigens, a single band ~1.7 kb in length was found for all family members (Fig. 2). Therefore, it appears that neither the genes nor the transcripts for class I and β_2-microglobulin are qualitatively abnormal in the bare lymphocyte syndrome.

Quantitative analysis by Northern dot blots was undertaken in order to evaluate the levels of class I and β_2-microglobulin transcripts in the circulating leukocytes from the affected and unaffected family members. 1 μg of total RNA isolated from the leukocytes of the family members was dotted onto nitrocellulose. This dot blot was then hybridized with either the class I, β_2-microglobulin, or an actin control probe (Fig. 3). Class I and β_2-microglobulin transcripts were found to be much lower by densitometry in the affected children than in the unaffected family members. Actin transcripts were only slightly decreased. Thus, the bare lymphocyte syndrome is associated with a quantitative defect in the number of class I and β_2-microglobulin transcripts.

Decreased levels of RNAs specific for class I and β_2-microglobulin could indicate a general diminution among all cells or represent the averaging of different levels among subpopulations of cells. The density of class I antigens on subpopulations of mononuclear cells was analyzed by flow cytometry. PBMCs from the affected family members stained with either anti-class I or anti-β_2-microglobulin monoclonal antibodies exhibited a density of each molecule that was 10–25% of that seen in unaffected family members or unrelated controls. Class I and β_2-microglobulin staining on platelets identified by forward light scatter demonstrated no difference between affected and unaffected family members (data not shown). Specific subpopulations of cells were identified by means of phycoerythrin-labeled antibodies (i.e., phycoerythrin Leu 1, phycoerythrin DR, and phycoerythrin M3), and the relative class I and β_2-microglobulin density on the cells of each subpopulation was determined by using FITC-linked antibodies (Fig. 4). In an affected child, class I and β_2-microglobulin-specific fluorescence associated with the T cell

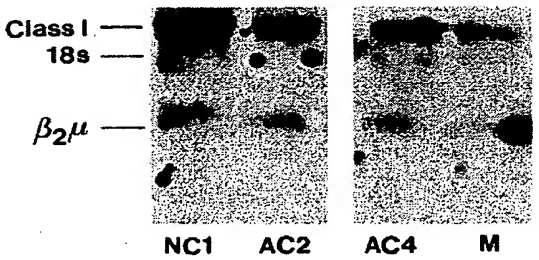

Figure 2. Northern blot of transcripts isolated from EBV-transformed cells of family "L." 25-μg samples of total RNA were electrophoresed on a Northern formaldehyde gel to assess transcript length differences in the affected children. Shown in this figure are bands hybridizing with class I and β_2-microglobulin (β_2 μ) specific probes for two unaffected family members (*M, NC1*) and two affected family members (*AC2, AC4*). There are no apparent differences in transcript length when affected are compared with unaffected family members.

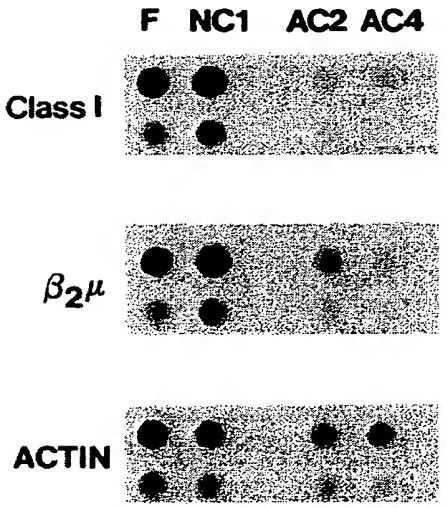

Figure 3. Quantitative analysis of class I and β_2-microglobulin transcripts. RNA was isolated as described from circulating leukocytes and dotted onto nitrocellulose in twofold dilutions of 1 μg of RNA. This blot was probed with class I, β_2-microglobulin (β_2 μ), or actin-specific probes as indicated.

population was 10.5-fold less than the mother or an unrelated control. Staining in the DR and M3 positive population was fourfold less in the affected child than in the control (Table I).

These findings suggest that not all cells are equally affected and that RNA levels might correspond more closely to surface expression if only affected cells are analyzed. When RNA from PBMCs, consisting of lymphocytes and monocytes, are analyzed by Northern blotting and densitometric scannings normalized to ribosomal (r)RNA levels, a 10-fold decrease of class I transcripts is seen in an affected child when compared to an unaffected family member (Fig. 5). These data demonstrate that the levels of class I and β_2-microglobulin transcripts correlate directly with the cell surface expression of these antigens. However, RNA dot blots revealed that class I and β_2-microglobulin transcripts are only threefold decreased in leukocytes, consisting of granulocytes in addition to lymphocytes and monocytes. This suggests that class I and β_2-microglobulin expression is not diminished in all circulating cells and that patients granulocytes may in fact have normal class I and β_2-microglobulin transcript levels.

Table I. Peak Fluorescence Intensity for PBMC Subpopulations

	Cell surface molecule detected		
Source	Leu 1	DR	M3
Unrelated control	112	110	105
Mother	89	85	89
AC2	8	22	24

In this table, values are given for the intensity of fluorescence corresponding to the β_2-microglobulin staining. Different subpopulations of cells were detected with a counterstain. Leu 1 depicts most T cells, DR depicts B cells and monocytes, and M3 depicts mature monocytes. Here, the intensity of β_2-microglobulin staining in the Leu 1 population can be seen to be much less in the affected child (AC 2) than in the mother (M). After normalization of peak channel values to background, values were converted from the logarithmic scale using the formula, $10^{x/57}$ = fluorescence intensity (x = peak channel value). Thus, in the Leu 1 population, the affected child's cells express only one-tenth the density of β_2-microglobulin as the mother. In the DR + and M3 population, the affected child's cells express approximately fourfold less β_2-microglobulin than the unaffected mother.

Discussion

These experiments have demonstrated a pretranslational defect in class I and β_2-microglobulin expression. This defect is differentially expressed in different subpopulations of cells and it is possible that only mononuclear cells are affected. When class I and β_2-microglobulin transcript levels are analyzed from total leukocytes they are found to be approximately threefold decreased. Mononuclear cell transcripts, on the other hand, are 10-fold decreased in the affected children. This suggests granulocyte expression may be normal in these children. Platelet expression was found to be normal and others have reported normal class I surface expression on fibroblasts, granulocytes, and platelets (1–6, 24).

Both class I and β_2-microglobulin-specific transcripts were found to be decreased in the mononuclear cells of these affected children. Because these genes reside on chromosomes 6 and 15, respectively, and because EBV reverses the defect, it is unlikely that mutations in either gene could account for our findings. Instead, it is likely that the bare lymphocyte syndrome represents a defect in the regulation of class I and β_2-microglobulin gene expression. A related syndrome with a class II defect is also thought to represent a defect in regulation (9).

That these genes share common regulatory pathways is suggested by the finding that they are coordinately expressed in development. Class I and β_2-microglobulin antigens are absent from fertilized mouse eggs but appear later in development (25, 26). This developmental control is reproduced in embryonal carcinoma cells (27). Embryonal carcinoma cells do not express class I antigens on their surface nor do they have detectable levels of transcript for either gene (28–30). Differentiation of these cells is accompanied by coordinate accumulation of messenger RNAs and surface expression of class I molecules (27). From these experiments, it is clear that class I and β_2 microglobulin genes are simultaneously induced during development.

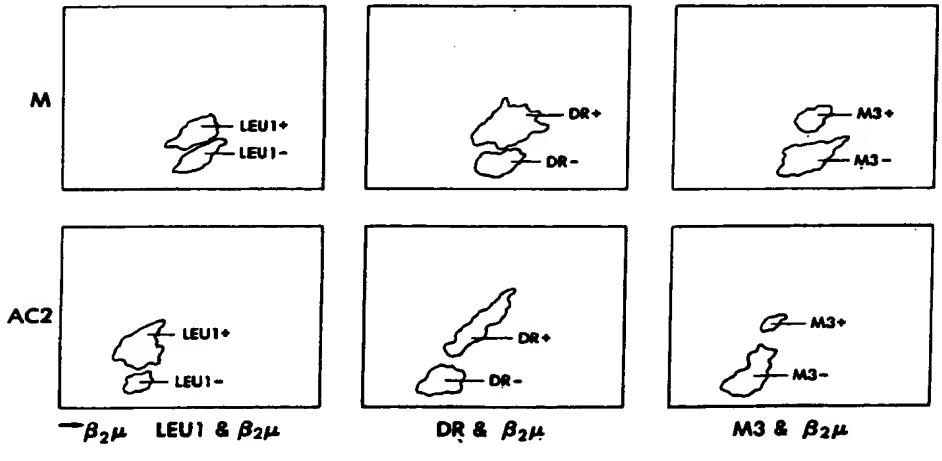

Figure 4. Two-color fluorescent cytometry analysis of β_2-microglobulin expression on PBMC subsets. This figure compares β_2-microglobulin staining of an unaffected family member's PBMC subsets (*M*) and those of an affected family member (*AC2*). The vertical axis depicts staining with either Leu 1, anti-DR, or M3 as indicated. Therefore, subsets are separated on the vertical axis, with the uppermost population staining positive with the indicated antibody. Notice that the intensity of flourescence in the vertical axis is normal in the affected child; demonstrating that Leu 1, DR, and M3 expression is normal on "bare lymphocytes." The horizontal axis depicts staining with anti-β_2-microglobulin. All subsets in the affected children exhibit decreased staining with anti-β_2-microglobulin with the greatest decrease apparent in T cells depicted by Leu 1. This is quantified in Table I.

DENSITOMETRY ANALYSIS

	M	NCI	AC2
CLASS I	8.8	6.3	1.0
ACTIN	7.5	5.2	3.5
RIBOSOMAL	4.5	3.1	5.1
CLASS I / RIBOSOMAL	1.95	2.05	0.19
ACTIN / RIBOSOMAL	1.67	1.67	0.69

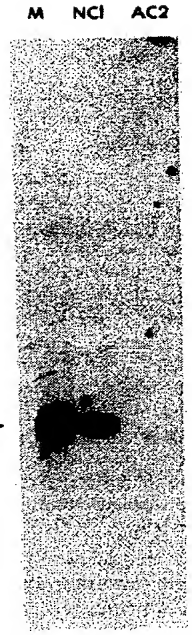

Figure 5. Northern blot and densitometry of transcripts isolated from the PBMCs (lymphocytes and monocytes) of three family members. Shown in this figure are bands hybridizing to the class I-specific probe from two unaffected family members (*M* and *NC1*) and one affected family member (*AC2*). Class I-specific transcripts are 10-fold less abundant in the affected child when normalized to ribosomal RNA levels. The densitometry table also reveals a slight decrease in actin transcript levels in the affected child. Notice the apparent difference in M and NC1 class I levels disappears when densitometry levels are normalized to correct for loading error.

Experiments investigating the regulation of expression of class I and β_2-microglobulin genes in a mature, expressing cell, also suggest that class I and β_2-microglobulin genes share common regulatory pathways. Lymphoblastoid and melanoma cell lines as well as PBMCs have been induced to increase their class I and β_2-microglobulin surface expression after administration of interferon (31–33). It is not known which sequences are responsible for the regulation of class I transcription. In transfection experiments, interferon was shown to increase the expression of the introduced HLA B7 genes irrespective of deletions in the 5' flanking region and the first exon (34, 35).

These experiments suggest that class I and β_2-microglobulin genes share common regulatory pathways. The bare lymphocyte syndrome could represent a defect at some step leading to coordinate gene expression. First, it could represent an abnormality in the production of lymphokines (e.g., interferon) known to increase class I and β_2-microglobulin expression. In two studies, patients with a defect in expression of both class I and class II molecules, α-interferon was able to restore class I expression (4, 36). Addition of α-interferon to the cells of one of our patients failed to reverse the defect (data not presented), suggesting that the defect is not simply due to decreased production of α-interferon. Second, a synthetic or structural abnormality in the α- or β-interferon receptor could make "bare lymphocytes" refractory to those lymphokines. We have no data to address this issue. Third, intracellular effector molecules such as DNA binding proteins that recognize regulatory sites flanking the class I and β_2-microglobulin genes and serve to modify their gene expression may be defective. If tissue-specific regulators of transcription are shared between these genes, one could postulate a lesion in a single protein that binds those specific DNA sequences. *Trans*- acting regulatory factors have been postulated for class II histocompatibility genes (37), and it is possible that they exist for class I histocompatibility genes. The low levels of class I and β_2-microglobulin transcripts, which are found in the lymphocytes of our patients, may be products of a constitutive pathway unaffected by the absence of a regulatory protein. This constitutive pathway may also account for the normal levels of class I and β_2-microglobulin found on our patients platelets.

The levels of class I and β_2 microglobulin transcripts in the bare lymphocytes were ~10-fold lower than in normal controls. The levels of surface class I molecules were from 4- to 10-fold diminished when affected and unaffected family members were compared. Actin levels were slightly decreased compared to controls and this may reflect altered metabolism in these cells. Therefore, a pretranslational defect in class I and β_2-microglobulin certainly constitutes a major factor in the bare lymphocyte syndrome, but at this time we are not able to rule out other effects. It should be stressed that the defect observed in this family with the bare lymphocyte syndrome without immunodeficiency may not reflect that found in other cases of the bare lymphocyte syndrome with immunodeficiency. Only through further study of this and similar disorders will the true nature of the bare lymphocyte syndrome defect be revealed and therapeutic intervention become possible.

Acknowledgments

We gratefully acknowledge the cooperation of the "L" Family in these studies. Contributions of probes by Dr. Itakura (City of Hope Research Institute), Dr. Weissman (Yale University School of Medicine), Dr. Gunning (Stanford University School of Medicine), and Andree Dozy of our institution are also acknowledged. Flow cytometry was performed on the Howard Hughes Medical Institute FACS IV with the technical assistance of Ms. Araxy Missirian-Bastian.

This work was support in part by The Council for Tobacco Research grant no. 1593, U. S. Public Health Service grant 5R01 AI14104, and National Institutes of Health National Institute of General Medical Services training grant GMO 7618.

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Expression of Ia-like antigens on cultured human malignant melanoma cell lines

(human cancer/differentiation antigen/alloantigen/B lymphocyte/glycoprotein)

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ABSTRACT **Human malignant melanoma cell lines were found to bear Ia-like cell surface determinants demonstrable by hetero- or alloantisera and by direct identification of the characteristic bimolecular glycoprotein complex. Immunoprecipitation confirmed the presence of Ia determinants on the bimolecular complex. The quantity of Ia molecules determined by these methods and by absorption experiments was relatively constant for each cell line. Among different lines, however, the amount of Ia antigens ranged from a level equal to that expressed by B-cell lines to a small fraction of this amount. This variation in level of Ia contrasted with the more uniform amount of β_2-microglobulin detected on the cell surface. The Ia alloantigen specificity DRw2 was the most frequently encountered specificity. Ia determinants were also found on the surface of an epidermoid carcinoma line, but not on various other cell lines of normal or neoplastic origin.**

Human B lymphocytes contain a polymorphic bimolecular glycoprotein component, encoded by genes mapping within the major histocompatibility complex, that is distinct from the classic HLA molecules. Because this molecular system has a number of characteristics shared by the murine I-region-associated antigens, it has been termed "Ia-like" or, for simplicity, "Ia." The selective occurrence of Ia molecules on the surface membranes of normal or malignant cells of the hematopoietic and lymphoid systems depends upon the differentiation state of the particular cell lineage. In the erythroid and granulocyte series only the progenitor cells and the earliest morphologically recognizable forms bear Ia antigens (1, 2). In contrast, B lymphocytes express Ia determinants throughout most of their differentiation sequence, the antigens becoming undetectable only during transition into plasma cells (3). Within the monocyte lineages the expression of Ia antigens is more variable, with a considerable proportion of circulating monocytes lacking detectable Ia determinants (4). In contrast, the majority of T lymphocytes, whether normal or malignant, have little or no detectable Ia.

In view of the finding that murine epidermal cells contain Ia determinants (5), considerable attention has been directed to a search for Ia determinants on human skin and its malignant derivatives. Recently Ia antigens in humans and guinea pigs were reported to occur on the Langerhans cell, a cell present in normal skin that is considered to be monocytoid in origin; the remaining epidermal cells, including melanocytes, did not express Ia antigens (6, 7). The present study was directed primarily at determining the occurrence of Ia antigens on various cell lines derived from different malignancies. The chief finding was the widespread occurrence of these molecules on malignant melanoma lines.

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MATERIALS AND METHODS

Melanoma and Other Adherent Cell Lines. Established cell lines were maintained in medium RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units of penicillin per ml, and 100 μg of streptomycin per ml (medium A). The cultures were fed twice weekly and passed when confluent at 7- to 14-day intervals. Passage and harvesting routinely involved brief (0.5–1 min) treatment with 0.01% trypsin (Bacto-Trypsin, Difco) at room temperature. The digestion was terminated by addition of 25 ml of medium A per ml of trypsin solution. The cells were washed once and either transferred to new flasks or adjusted to 1×10^7 cells per ml of medium A and incubated at room temperature for 1 hr prior to subsequent examination. The melanoma cell lines were established by described methods (8) from tumors obtained from patients with stage II or III disease. The two renal carcinoma cell lines were established by R. Ueda and H. Shiku. The origin of the other lines has been described (9).

Lymphoid Lines. B-type lymphoblastoid lines were initiated from 20 normal individuals of defined HLA-A, B, C, and D/DR type. Transformation was induced by supernates containing Epstein–Barr virus obtained from the marmoset lymphoblastoid line, B95-8. The B cell line, B35M, and the T cell lines, 1301 and CEM-T, were provided by J. Minowoda. All cell lines were maintained in medium A. Before use they were washed four times with Dulbecco's saline solution.

Heteroantisera. The Ia antiserum was prepared by immunizing rabbits with a 65,000-dalton bimolecular glycoprotein complex isolated from the membranes of B35M that has the Ia phenotype DRw3, $4 \times 7 \times 10$. The 65,000-dalton component contained B35M Ia alloantigens and dissociated to 28,000- and 37,000-dalton polypeptides upon denaturation at 100°C. Details of preparation and characterization of the antiserum have been described (10). An antiserum to β_2-microglobulin was provided by S. M. Fu and R. Stern.

Ia Specific Alloantisera. Ia (DR) specific alloantisera were obtained from individuals immunized during pregnancy or by organ transplantation, and selected as described (11).

Immunofluorescence. Heteroantisera were used in direct fluorescence after digestion to $F(ab')_2$ fragments and conjugation with tetramethylrhodamine isothiocyanate as described (11). The reactivity of the alloantisera was determined by indirect immunofluorescence with $F(ab')_2$ fragments of goat anti-Fc fragment of IgG conjugated with tetramethylrhodamine isothiocyanate. The intensity of surface immunofluorescence was graded 0, tr, 1, 2, 3, or 4 on the basis of the reaction of the majority of cells in a cell population.

Immunoperoxidase. The method of Nakane and Kawaoi (12) as modified (13) was used to conjugate horseradish peroxidase to $F(ab')_2$ fragments of heteroantisera to Ia.

Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

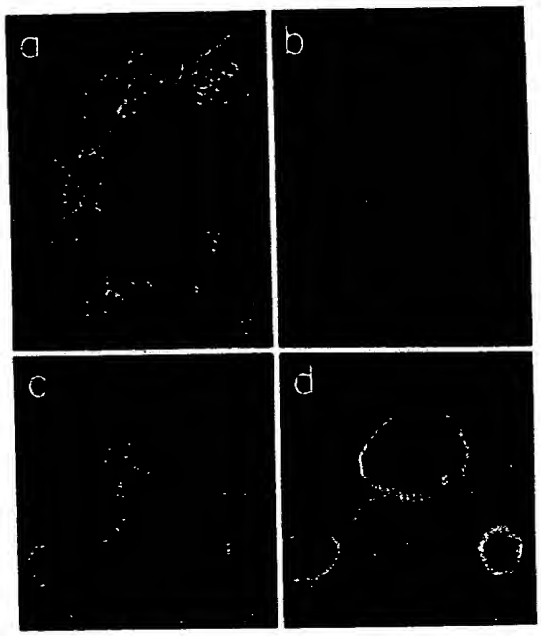

FIG. 1. Presence of Ia antigens on the surface of melanoma cell lines as demonstrated by hetero-anti-Ia sera in direct immunofluorescence tests. (*a* and *c*) Phase micrographs of SK-Mel-13 and SK-Mel-37 cells, respectively. (*b* and *d*) Identical field under illumination for fluorescence microscopy.

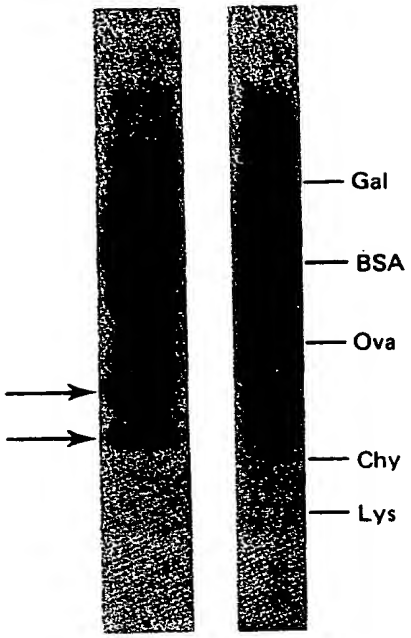

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis fluorogram of (*Left*) SK-Mel-37 and (*Right*) SK-Mel-33 labeled by the neuraminidase–galactose oxidase–boro[^{3}H]hydride procedure. Arrows indicate the bimolecular complex (gp 37,28) labeled in SK-Mel-37 but not in SK-Mel-33. Molecular weight standards: Gal, β-galactosidase (120,000); BSA, bovine serum albumin (68,000); Ova, ovalbumin (45,000); Chy, α-chymotrypsinogen (25,700); Lys, lysozyme (14,300).

Absorption Tests. The hetero-anti-Ia reagent, conjugated with rhodamine, was diluted 1:10 for absorption tests (dilution determined by preliminary experiments). Samples (0.05 ml) of the diluted Ia reagent were added to microfuge tubes containing the appropriate quantities of pelleted cells from which the supernatant fluid was removed. The mixture of cells and reagent was suspended and incubated for 30 min at 37°C.

After removal of the cells by centrifugation, 0.025 ml of the supernate was used to stain a B-cell line. In order to avoid interference by heteroantibodies with specificities for Ia alloantigens, the B-cell line used for the previously mentioned staining assay was selected because its Ia phenotype (D/DRw1) did not share Ia alloantigens with the cell line used as the immunogen. B-cell absorption was performed with a pool of 20 B-cell lymphoblastoid lines.

Immunoprecipitation. The surface membranes of melanoma cell lines were labeled with ^{125}I, and solubilized membrane preparations were obtained with Nonidet P-40 as described (10). Immunoprecipitations with the hetero-anti-Ia serum were performed with protein A-Sepharose CL-4B beads as a solid phase absorbant; the immunoprecipitates were analyzed by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate ($NaDodSO_4$) (14–16).

Carbohydrate-Labeling Procedures. Monolayer cells were labeled *in situ* in T-75 plastic tissue culture flasks after the cultures had become fully confluent. The procedure used was the neuraminidase–galactose oxidase boro[^{3}H]hydride method of Gahmberg and Hakomori (17). After the cells were labeled, the components were examined by 5–15% gradient, polyacrylamide slab gel electrophoresis in 0.1% $NaDodSO_4$ (16) with subsequent fluorography (18). The quantity of glycoprotein with mobilities characteristic of Ia molecules was determined by densitometric scan of the radiofluorograms and expressed

Table 1. Expression of Ia antigens and gp 37,28 on melanoma cell lines

	β_2-Microglobulin direct immunofluorescence		Ia direct immunofluorescence		gp 37,28,
Cell line	Relative intensity	Positive cells, %	Relative intensity	Positive cells, %	% of total glycoprotein*
SK-Mel-13	2–4	100	3–4	100	3.7
SK-Mel-37	3–4	100	3–4	100	16.7
SK-Mel-42	tr–1	100	2–4	100	7.8
SK-Mel-29	2–4	100	tr–3	100	<2
SK-Mel-33	1–3	100	tr–3	100	<1
SK-Mel-41	tr–1	100	tr–3	100	ND
SK-Mel-28	2–4	100	tr–3	35	<1
MeWo	2–3	100	tr–1	24	<2
SK-Mel-19	2–4	100	tr–1	3	<2
SK-Mel-27	1–2	100	tr–1	2	<1

* Determined by carbohydrate-labeling procedure. ND, not determined.

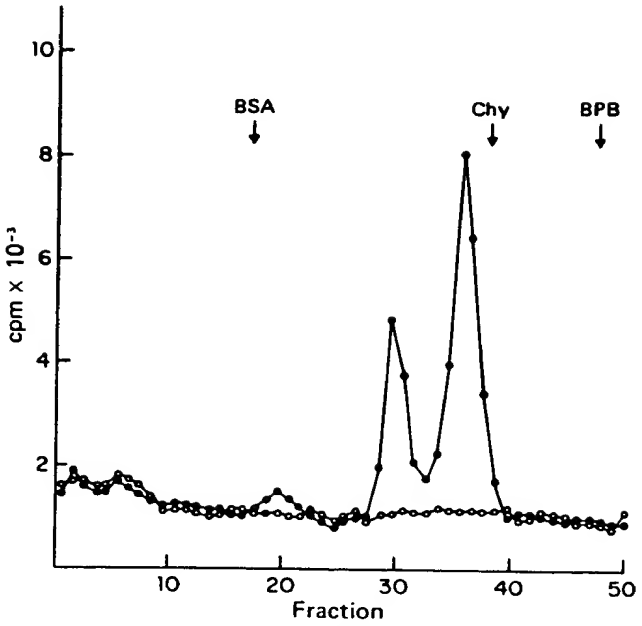

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis of ^{125}I-labeled antigens of (●) SK-Mel-37 and (○) SK-Mel-19 immunoprecipitated with rabbit anti-Ia serum. SK-Mel-37 has the gp 37,28 bimolecular complex characteristic of Ia molecules; the complex was not detected by this method in SK-Mel-19. Molecular weight standards: BSA, bovine serum albumin (68,000); Chy, α-chymotrypsinogen (25,700); BPB, bromophenol blue (dye front).

as a percentage of the total ^{3}H-labeled membrane glycoproteins.

RESULTS

The intense reaction of heterologous anti-Ia serum with two melanoma cell lines, SK-Mel-13 and SK-Mel-37, is illustrated in Fig. 1. The pattern of fluorescent staining was continuous, with only occasional cells showing a speckled granular distribution of the fluorescent reagent. Each cell in the culture was stained; the vast majority of cells reacted with a relative intensity of 3+ to 4+, comparable to the brightest staining of B-cell lymphoid lines. An occasional cell gave 1+ to 2+ staining.

Table 1 illustrates the range of reactivity with the heterologous anti-Ia serum that characterized the different melanoma cell lines. Of the ten lines studied, six had Ia determinants detected on all cells of the line; two, SK-Mel-28 and MeWo, had intermediate numbers of definitely stained cells; two other lines, SK-Mel-19 and SK-Mel-27, had very low percentages of cells showing staining with the Ia reagent. With the latter four cell lines, the positive cells varied considerably in staining intensity. In each instance, however, staining was specifically removed by absorbing the anti-Ia sera with pooled B-cell lines but not with T-cell lines. Among the cells lacking definite staining (equal or greater than tr) was a significant proportion of cells that had very weak, vague staining reactions of insufficient intensity to be reliably scored as positive. Table 1 also shows the relation between Ia detected serologically and glycoproteins, detected by a carbohydrate-labeling technique, with molecular weights of 37,000 and 28,000 characteristic of Ia molecules designated gp 37,28. The three lines (SK-Mel-13, -37, and -42) that had the greatest amount of Ia detected by immunofluorescence had readily detected gp 37,28 (Fig. 2). In melanoma cell lines expressing smaller amounts of Ia, it was not possible to identify the characteristic bimolecular complex by carbohydrate labeling. The Ia bimolecular complex could also be demonstrated by surface radiolabeling with ^{125}I and subsequent immunoprecipitation with the heterologous anti-Ia reagent (Fig. 3). The level of Ia antigen demonstrated by this method paralleled results obtained with direct immunofluorescence (Table 1). β_2-Microglobulin could be detected on all the melanoma cell lines tested (Table 1), and the variation in amount of Ia antigen contrasted with the more uniform expression of β_2-microglobulin.

The intensity of Ia staining appeared to be a relatively stable feature of the various melanoma cell lines tested. For example, SK-Mel-37 and SK-Mel-13 had essentially identical reactions in tests of cultures separated by over 20 passages. The percentage of positive cells in SK-Mel-28 and MeWo varied between 15 and 39%, while SK-Mel-19 and SK-Mel-28 always had less than 5% Ia positive cells. The effect of trypsin digestion, used routinely to harvest cells for serological study, was evaluated by comparing enzyme-treated cells with cells mechanically detached from the flask surface by scraping. No difference in Ia-staining reactions was found with melanoma cells prepared by these two methods.

Absorption tests were performed to investigate the serological

Table 2. Quantitative absorption tests of hetero-anti-Ia serum: Comparison of Ia expression on B-cell lines and melanoma cells*

Cell type used for absorption	No. of absorbing cells/0.05 ml antiserum†					
	None	1×10^5	5×10^5	1×10^6	1×10^7	2×10^7
B-cell line pool	1–3 100%	0	0	0	0	0
SK-Mel-37	1–3 100%	0	0	0	0	0
SK-Mel-29	1–3 100%	tr–1 100%	tr 55%	0	0	0
SK-Mel-27	1–3 100%	1–3 100%	tr–2 100%	tr–2 100%	tr–1 100%	tr 100%
T-cell lymphoid line pool	1–3 100%	1–3 100%	1–3 100%	1–3 100%	1–3 100%	1–3 100%

* The hetero-anti-Ia reagent was used at a 1:10 dilution. The residual anti-Ia activity was assayed on a B-cell line of Ia phenotype D/DRw1.

† Relative intensity and percentage of stained designated cells.

Table 3. Representative reactions of Ia alloantisera with melanoma cell lines detected by indirect immunofluorescence

Cell line	Ia reagent specificity*					
	DRw2	DRw3	DRw 4 × 7 × 10	DRw5	Multi "Ve"	Multi "Pr"
SK-Mel-37	tr–3 96%	0	0	0	tr–4 100%	0
SK-Mel-29	tr–2 92%	0	tr–2 96%	0	tr–3 92%	tr–4 10
SK-Mel-27	3 2%	0	2 4%	0	0	0

* Relative intensity and percentage of the designated cells stained in indirect immunofluorescence.

relatedness of Ia determinants on melanoma cells and on B-cell lines and to provide a quantitative estimate of the Ia-antigen expression on the melanoma lines (Table 2). Both SK-Mel-37 and SK-Mel-29 cells removed all Ia reactivity for the B-cell lines from the heteroantibody, indicating that the spectrum of Ia determinants expressed by melanoma cells was qualitatively similar to those expressed on B cells. Quantitative absorption analysis with three melanoma lines showed that absorption capacity paralleled the results of direct fluorescence tests. Absorption with 1×10^5 SK-Mel-37 cells removed all Ia antibodies, whereas 1×10^6 SK-Mel-29 cells were required to achieve the same endpoint. Absorption with SK-Mel-19 and SK-Mel-27, two of the weakest reactors in direct tests with Ia antisera, required 2×10^7 cells before Ia reactivity was diminished.

The expression of Ia alloantigens on the melanoma cell lines was studied with pregnancy or transplant reagent antisera; representative tests are shown in Table 3. These alloantisera gave a complex pattern of positive and negative reactions with the different melanoma cell lines. With melanoma lines that reacted strongly with the heterologous anti-Ia sera (e.g., SK-Mel-37), Ia alloantigens could be detected by cytotoxic and indirect fluorescent assays, and parallel results were given by both methods. In contrast, lines such as SK-Mel-27, with very low reactivity with Ia heteroantibody, showed no sensitivity to cytotoxic Ia antibody. However, in immunofluorescence tests, bright staining of a low percentage of cells was given by certain alloantisera, comparable to results with the heterologous anti-Ia sera. Typing with a battery of Ia reagents showed that the most frequent Ia alloantigen associated with these melanoma cultures was the specificity DRw2, detected on six of eight melanoma lines.

Five metastatic melanoma tumors, excised from patients, were studied for the occurrence of Ia antigens by Daniel M. Knowles. The immunoperoxidase method was used on thin sections of paraffin-embedded tumor. Four of the five tumors had definite strong staining which was not found when the anti-Ia reagent was first absorbed by pooled B cells. One tumor had a level of staining only slightly greater than the absorbed control serum, and a definite conclusion regarding the presence or absence of Ia determinants was not possible.

Table 4 lists the 13 non-melanoma cell lines of human origin that have also been tested for Ia and β_2-microglobulin. The Hep 2 line, originally derived from an epidermoid carcinoma of the larynx, was found to express Ia determinants. All Hep 2 cells were stained, and the intensity of staining varied from 1+ to 2+. Ia antigen was not detected on the other cell lines. Direct carbohydrate labeling did not detect gp 37,28 molecules on any of these cell lines, including Hep 2.

DISCUSSION

Detailed knowledge of the surface composition of melanoma cells may provide some insight into the unusual biological features of this malignancy. Most emphasis has been on the search for melanoma-specific cell-surface antigens, and evidence for the existence of such antigens is growing stronger. Less attention has been given to the total antigenic phenotype of melanoma cells, and the list of normal antigens expressed on melanoma cells is restricted to certain A and B locus HLA determinants that are associated with β_2-microglobulin. In the present study, the existence of Ia antigens on the surface of cultured human melanoma cells was documented in several ways: (*i*) parallel reactivity of both allo- and heterologous anti-Ia sera; (*ii*) occurrence of Ia antigens on the characteristic bimolecular complex of 28,000 and 37,000 daltons by immunoprecipitation; (*iii*) direct demonstration of two glycoproteins with appropriate molecular weights by cell-surface carbohydrate-labeling procedures; and (*iv*) absorption tests that pro-

Table 4. Presence of Ia determinants on various cultured human cell lines

Cell line	Tissue derivation	Relative intensity and % of cells expressing*	
		β_2-Microglobulin	Ia
Hep 2	Epidermoid carcinoma	2–4, 100%	1–2+, 100%
U 205	Sarcoma	tr–1, 100%	0
SK-RC-1	Renal carcinoma	tr–1, 100%	0
SK-RC-6	Renal carcinoma	4, 100%	0
J82	Astrocytoma	1–2, 100%	0
T24	Bladder carcinoma	3+, 100%	0
SK-LC-LL	Lung carcinoma	2, 100%	0
ME 180	Cervical carcinoma	3, 100%	0
WI 38	Fetal lung fibroblasts	2, 100%	0
Gm 43	Normal fibroblasts	3, 100%	0
ALAB	Breast carcinoma	±–tr, 100%	0
SK-Lu-1	Lung carcinoma, undifferentiated	1, 100%	0
SK-OV-3	Ovarian carcinoma	2, 100%	0

* Determined by direct immunofluorescence with the hetero reagents, and expressed as relative intensity and percentage of staining of the designated cells.

vided further evidence for the similarity of the Ia determinants on melanoma cell lines to those on B lymphoid cell lines.

One technical aspect of this study that requires comment is the use of tyrpsin treatment to remove the adherent melanoma cells. No evidence was obtained that suggested diminution of the expression of Ia determinants under the conditions of minimal digestion. The use of mechanically removed cells in the absence of exposure to enzyme gave essentially equivalent results and suggested that the Ia antigens were not present as cryptoantigens on melanoma cells, requiring enzymatic digestion to be revealed. Moreover, Ia molecules were detectable on nontrypsinized monolayer cells by a procedure that directly labels cell-surface carbohydrates. A second consideration has to do with the unlikely possibility that the Ia-positive cells in melanoma cultures were cocultured non-melanoma cells such as B lymphocytes arising from lymphocytes in the original explant. Phase microscopy revealed that Ia-staining cells were typical epithelioid adherent cells containing melanin. Furthermore, the presence of Ia antigens on freshly excised tumors also strongly supports the finding of Ia determinants on cultured cell lines.

The fact that six of the eight melanoma cell lines reacted with alloantisera specific for Ia-2 or DRw2 raises the possibility of a common immunogenetic predisposition to melanoma development. Although the nature of this study does not permit a conclusion in this matter, it is of interest that DRw2 was present in 28% of a normal New York population by B-cell typing and is a constituent of the HLA-A3-B7-Dw2-DRw2 haplotype that is characteristic of northern European populations. Epidemiologic evidence has demonstrated a higher frequency of melanoma in individuals of Northern European ancestory.

The level of Ia antigens expressed on the different melanoma cell lines showed wide variation. Evidence from absorption tests indicated that the average quantity of Ia antigens varied by more than 200-fold among the different melanoma cell lines. This variability was a characteristic property of the cell line, and the extent to which a line expressed Ia antigens differed only slightly in multiple examinations over an extended period of time.

A likely possibility to account for the variable pattern of Ia expression on different melanoma lines is that it reflects similar variation in the expression of Ia antigens on normal melanoblasts or melanocytes during differentiation and maturation. By analogy with the findings in granulocytic leukemia, melanoma cell cultures with the greatest quantity of Ia antigens would represent cells transformed at an early stage in the melanocyte lineage. An important point for further study is whether the high, intermediate, or low Ia phenotype of melanoma cells shows any correlation with clinical features of the disease, such as stage, growth rate, or metastatic behavior.

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Lack of HLA Class I Antigen Expression by Melanoma Cells SK-MEL-33 Caused by a Reading Frameshift in β_2-Microglobulin Messenger RNA

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Abstract

The lack of HLA class I antigen expression by the melanoma cell line SK-MEL-33 is caused by a unique lesion in β_2-microglobulin (β_2-μ). Sequencing of β_2-μ mRNA detected a guanosine deletion at position 323 in codon 76 that causes a frameshift with a subsequent introduction of a stop codon at a position 54 base upstream of the normal position of the stop codon in the message. The loss of 18 amino acids and the change of 6 amino acids, including a cysteine at position 80 in the carboxy terminus of β_2-μ, are likely to cause marked changes in the structure of the polypeptide. The latter may account for the inability of β_2-μ to associate with HLA class I heavy chains and for its lack of reactivity with the anti-β_2-μ mAb tested. HLA class I antigen expression on SK-MEL-33 cells was reconstituted after transfection with a wild-type B_2m gene, therefore indicating that the abnormality of endogenous B_2m gene is the only mechanism underlying lack of HLA class I antigen expression by SK-MEL-33 cells. The guanosine deletion in B_2m gene was detected also in the melanoma tissue from which SK-MEL-33 cells had originated. Therefore, the molecular lesion identified in the SK-MEL-33 melanoma cell line is not caused by a mutation acquired during growth in vitro but is likely to reflect a somatic mutation during tumor progression. (*J. Clin. Invest.* 1993. 91:684–692.) Key words: β_2-microglobulin sequencing • transfection • HLA reconstitution • monoclonal antibody

Introduction

Malignant transformation of human melanocytes may be associated with changes in their antigenic profile. The latter include reduction or loss of HLA class I antigen expression, which has been found to occur in ~ 30% of surgically removed melanoma lesions (for review, see reference 1). Abnormalities in HLA class I antigen expression are likely to affect the biology of melanoma cells and their interactions with immune cells because of the role of HLA class I antigens in cell proliferation (2–4), interaction of target cells with cytotoxic T cells (for review see reference 5), and susceptibility of malignant cells to natural killer (NK)[1] cell–mediated lysis (for review see reference 6). Besides contributing to the characterization of the machinery regulating HLA class I antigen synthesis and expression, analysis of the molecular mechanisms underlying reduction or loss of HLA class I antigen expression by melanoma cells provides the necessary background to correct these abnormalities. This information may eventually be used to develop therapeutic approaches to melanoma, if the association between poor prognosis and reduction or lack of HLA class I antigen expression in melanoma lesions (7) reflects their role in the clinical course of the disease.

In a recent study we have shown that melanoma cells FO-1 do not express HLA class I antigens because of a gross deletion of the 5′ region and a portion of the coding sequence of the B_2m gene that prevents its transcription (8). In the present study, we have characterized the molecular defect that accounts for the lack of HLA class I antigen expression by melanoma cells SK-MEL-33.

Methods

Patient. Patient AZ was a 74-yr-old white male who had a lentigo maligna melanoma excised from the skin of his right scapula in 1972. Two years later, in January 1974, a mass appeared in the left axilla. The mass was excised and revealed metastatic melanoma involving regional lymph nodes. A large (5 × 4 × 4 cm), firm mass reappeared at the apex of the left axilla in June 1974. The patient underwent a deep axillary lymph node dissection. Pathological examination showed metastatic melanoma within lymph nodes invading the surrounding subcutaneous tissue, fat, pectoralis minor muscle, brachial nerve plexus, and brachial vein. The melanoma cell line SK-MEL-33 was established from this lesion. The patient remained free of any recurrent melanoma for ≥ 10 yr. He was last seen for follow-up in 1983 when he was 85 yr old. Clinical staging at that time demonstrated no evidence of recurrent melanoma on physical examination, chest x ray, and liver function tests.

Cell lines, tumor specimen, and β_2-microglobulin (β_2-μ). The melanoma cell line SK-MEL-33 was cultured in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS, 2 mM L-glutamine, and 1% nonessential amino acids. The melanoma cell lines FO-1, Colo 38 and 3S5 and the B lymphoblastoid cell line MANN were grown in RPMI 1640 medium supplemented with 10% Serum Plus (Hazelton Biologics, Inc., Lenexa, KS) and 2 mM L-glutamine. Cells were harvested by vigorous pipetting with PBS supplemented with 1 mM EDTA.

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1. *Abbreviations used in this paper:* β_2-μ, β_2-microglobulin; ICAM-1, intercellular adhesion molecule-1; NK, natural killer.

The formalin-fixed and paraffin-embedded specimen of the human malignant melanoma lesion (Clark's level IV), from which the cell line SK-MEL-33 was originally established, was retrieved from the files of the Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York. The tumor had been fixed in 10% formalin (formaldehyde 4% vol/wt, methyl alcohol 1% vol/wt, buffered with phosphate buffer) (Mallinckrodt Chemical Works, NY) for 24 h before embedding into paraffin. One section was cut for standard hematoxylin and eosin staining and three adjacent 20-μm thick sections containing only tumor were cut for DNA isolation. Lesional portions of the two unstained sections were scraped with a stainless steel spatula into a 1.5 ml Eppendorf tube.

β_2-μ was purified from urine as described (9).

mAb and conventional antisera. The mAb W6/32 to a monomorphic determinant expressed on β_2-μ–associated HLA class I heavy chains (10); the mAb TP25.99 to a determinant expressed on both β_2-μ–associated and β_2-μ–free HLA class I heavy chains (8); the anti–human β_2-μ mAb BBM-1 (11), L368 (12), and NAMB-1 (13); the anti-HLA-DR, DQ, DP mAb Q5/13 (14); and the antiintercellular adhesion molecule-1 (ICAM-1) mAb CL207.14 (15) were developed and characterized as described. mAbs were purified from ascitic fluid by sequential precipitation with caprylic acid and ammonium sulphate (16). mAbs were labeled with ^{125}I using the iodogen method (17). The immunoreactive fraction of the radiolabeled antibodies was at least 50% as determined by the method of Lindmo et al. (18).

The rabbit antiserum R5996-4 reacting with denatured HLA class I heavy chain and the rabbit anti-human β_2-μ antiserum DP-213456 were prepared by following the methodology described by Nakamuro et al. (19) and by Poulik et al. (20), respectively. Anti–human β_2-μ antibodies were purified from rabbit antiserum DP-213456 by affinity chromatography on purified β_2-μ coupled to AFFI-GEL 10 (Bio-Rad Laboratories, Richmond, CA) (1 mg/ml of gel). Purified goat anti–rabbit Ig antibodies and rabbit anti–mouse Ig antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (Avondale, PA).

cDNA probes, B_2m gene, and oligonucleotides. The cDNA probes for human β_2-μ (21) and for HLA-B7 antigen (22) were isolated by digestion of plasmids with restriction endonuclease PstI. The inserts were electrophoresed and excised from low melting agarose gel. Human B_2m clone pb2m13 in the vector pEMBL9 (23) was purified on a CsCl gradient and digested either with XbaI alone or with the combination of HindIII and XbaI or of EcoRI and SmaI. An upstream 5' fragment (referred to as probe A), the first exon and much of its flanking sequences (referred to as probe B), the second and third exons and their flanking sequences (referred to as probe C), and the fourth exon and its flanking sequences (referred to as probe D) were separated by electrophoresis on a low melting point agarose gel as described (8). cDNA probes were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) by random priming (24) to a specific activity of 10^8 cpm/μg. Oligonucleotides were end labeled with [γ-^{32}P]ATP (5,000 Ci/mmol) in the presence of T4 polynucleotide kinase (25).

Oligonucleotides specific for the B_2m gene were synthesized on a DNA synthesizer (BioSearch, MilliGen/Biosearch, A Div. of Millipore, Burlington, MA). A 23-nucleotide oligomer (5' TAGAAAGACCAGTCCTTGCTGAA 3') and a 21-nucleotide oligomer (5' TGTATAAGCATATCAATATTA 3') complementary to the codon 56–63 and position 467–486 in the 3' untranslated region of human β_2-μ mRNA, respectively, were used as primers for RNA sequencing. The nucleotide oligomers (5' GTGGAGCATTCAGACTTGTC 3' referred to as primer 1 and 5' GCAGTGCCACTAATCTGATC 3' referred to as primer 2), which define a region of 300 bp, including codons 49–95 in the second exon of the B_2m gene, were used as primers for PCR. One oligomer (5' GCATACTCATTTTTTCAGTG 3') complementary to codon 72–79 in exon 2 of wild-type B_2m gene with a cytosine deletion at the region complementary to codon 76, referred to as probe II, and the other one (5' TAAGGCCACGGAGCGAGACAT 3') complementary to codon 1–7 in exon 1 of wild-type B_2m gene, referred to as probe I, were used for DNA hybridization.

Chemicals and cytokines. Actinomycin D was purchased from Sigma Chemical Co., (St. Louis, MO). Recombinant human IFN-γ was obtained from Hoffman-LaRoche, Inc. (Nutley, NJ).

Serological assays. The direct binding assay was performed as described (18) in 96-well microtiter plates (Becton Dickinson Co., Oxnard, CA). Briefly, cells (1×10^5) were incubated with ^{125}I-labeled mAb (2×10^5 cpm per well) at 4°C for 2 h. Then cells were washed five times with PBS and cell bound radioactivity was measured in a gamma counter (LKB-1261; LKB-Wallac, Turku, Finland). Results are expressed as bound cpm per 1×10^5 cells.

Radiolabeling of cells, indirect immunoprecipitation, and SDS-PAGE. These procedures were performed as described elsewhere (8). Cells were labeled with ^{125}I (Amersham Corp.) using the lactoperoxidase method (26) or with [^{35}S]methionine (Amersham Corp.). Then cells were solubilized by incubation for 30 min at 4°C in lysis buffer containing 1% Nonidet P-40, 10 mM Tris-HCl, pH 8.2, 0.5 M NaCl, 1 mM EDTA, 1 mg/ml BSA, and 1 mM PMSF and incubated for 12 h at 4°C with antibodies bound to protein A Sepharose (Pharmacia Inc., Piscataway, NJ). One-dimensional SDS-PAGE analysis was performed under reducing conditions in slab gels containing 12.5% acrylamide and using the buffer system described by Laemmli (27). Gels containing ^{125}I-labeled samples were processed for autoradiography using Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY). Gels containing [^{35}S]methionine-labeled samples were processed for fluorography as described (28).

Preparation of total mRNA and poly A$^+$ RNA. Total RNA was isolated from cells using the method described by Geliebter et al. (29). Poly(A)-containing RNA was purified by chromatography on oligo(dT) cellulose (Boehringer Mannheim Corp., Indianapolis, IN).

Preparation of DNA from paraffin tissue sections. Tissue sections were incubated in xylene to dissolve the paraffin. The tissue was then pelleted by centrifugation at 12,000 *g* for 5 min (30, 31). The supernatant was discarded and after two additional incubations with xylene the pellet was washed sequentially with 100% and 70% ethanol. The pellet was resuspended in 100 μl of a digestion buffer (0.2 M Tris-HCl, pH 8; 10 mM EDTA, 1% SDS) to which Proteinase K stored at −20°C was added to a final concentration of 0.5 mg/ml. At the end of a 48-h incubation at 55°C, more SDS and freshly thawed Proteinase K were added to the reaction mixture to a final concentration of 2% and 1 mg/ml, respectively. Incubation was continued for an additional 25 h at 55°C. The sample was then heated to 95°C for 6 min to inactivate Proteinase K and was extracted twice with phenol–chloroform. Finally, DNA was precipitated in 0.3 M NaCl and 100% ethanol at −70°C for 12 h and pelleted by centrifugation at 12,000 *g* for 30 min. The pellet was washed twice with 70% ethanol and resuspended in 100 μl of filtered and autoclaved water.

RNA hybridization analysis. Total RNA was size fractionated in a 1% agarose gel containing 2.2 M formaldehyde, transferred to a nitrocellulose membrane, and hybridized with ^{32}P-labeled probes as described (8).

DNA hybridization analysis. Genomic DNA was extracted essentially as described by Perbal (32). DNA was digested with restriction endonucleases, size fractionated, transferred to nitrocellulose membrane, and hybridized as described previously (8). When ^{32}P-labeled specific oligonucleotides for the B_2m gene were used as probes, the hybridization was performed at a temperature 5°C below the calculated melting temperature as described (33).

RNA sequencing. β_2-μ mRNA was sequenced by following the methodology described by Geliebter et al. (25). Briefly, ^{32}P-labeled oligonucleotide primers (10 ng) and poly A$^+$ RNA (20 μg) were heated to 80°C for 3 min in 15 μl of annealing buffer (250 mM KCl, 10 mM Tris-HCl, pH 8.3, 1 mM EDTA) and incubated for 60 min at 60°C or at 45°C. The dideoxynucleotides diluted with dH_2O in different ratio and 3.3 μl of reverse transcriptase diluted in extension buffer (24 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, 8 mM dithiothreitol, 0.2 mM dATP,

0.2 mM dCTP, 0.2 mM dTTP, 0.2 mM dGTP) were added into RNA primer–annealing solution, mixed, and incubated for 60 min at 50°C. Reactions were terminated by adding 2 μl of loading buffer (100% formamide, 0.3% xylene cyanol F, 0.3% bromophenol blue). Samples were electrophoresed in 6% polyacrylamide, 8 M urea gel at 17–18 V. After electrophoresis, gels were dried and exposed to Kodak XAR-5 film (Eastman Kodak Co.) for 72 h at −70°C. Nucleotide sequences were translated into amino acid sequences by using the software microgenie (Version 7.0, Beckman Instruments, Inc., Fullerton, CA).

In vitro amplification of genomic DNA. Extracted DNA was used as a template for amplification using the Thermus aquaticus (Taql) heat-stable DNA polymerase as described (34). Amplifications were performed in siliconized tubes (Bio-Medica, Rutherford, NJ) in a 50-μl solution containing genomic DNA template, 50 mM KCl, 10 mM Tris, pH 8.4, 2.5 mM $MgCl_2$, 300 ng of primer 1 and primer 2, 200 μM of each deoxyribonucleotide triphosphate (dATP, dCTP, dTTP, and dGTP), 100 μg of gelatin/ml, and 3 U of Taql DNA polymerase (Perkin-Elmer Cetus Instruments, Norwalk, CT). Samples were overlaid with 50 μl of mineral oil to prevent condensation and were subjected to 35 cycles of amplification. The cycling reaction was performed in a programmable heat block (DNA Thermal Cycler; Perkin-Elmer Cetus Instruments) set to heat samples to 94°C for 1 min, cool them to 50°C over 1 min, and heat them to 73°C for 1 min. In the first cycle DNA was denatured at 94°C for 5 min. Amplified DNA was analyzed by nondenaturing agarose gel electrophoresis. 5 μl of the amplification reaction was electrophoresed on a 1.5% Tris–Borate–EDTA agarose gel (FMC Corp., Rockland, ME) in the presence of ethidium bromide.

Cloning and sequencing of PCR products. B_2m PCR products were ligated into plasmid vector PCR (Invitrogen, San Diego, CA) and used to transform competent bacteria (*Escherichia coli* INVαF'). Single colonies of transformed bacteria were selected in medium containing kanamycin (50 μg/ml) and grown to mass culture. Amplified plasmid was extracted according to established protocols (35) and used in direct DNA sequencing reactions using the chain-termination technique (36) with a modified T7 DNA polymerase (37), (Sequenase 2.0; United States Biochemical Corp., Cleveland, OH). Plasmid DNA (2.5 μg) was incubated with 10 ng of M13(−)20 Primer-(5'-GTAAAACGACGGCCAGT-3') (Stratagene, Inc., La Jolla, CA) in 0.25 N NaOH at 20°C for 5 min. Denatured plasmid and annealed primer mixture were then precipitated with 5 M ammonium acetate, pH 4.6, and ethanol and were centrifuged at 15,000 *g* for 15 min. Pellets were washed with 70% ethanol, recentrifuged, dried, and resuspended in 8 μl of water. The annealed primer/template mixture was supplemented with 1 μl 0.1 M DDT, 1 μl 10 mU [α-^{35}S]dATP (1,000 Ci/mmol; New England Nuclear, Boston, MA), 2 μl dilute labeling mixture (1:5), and 3 U of Sequenase 2.0 (United States Biochemical Corp.). The reaction mixture was mixed, incubated for 2 min at room temperature, and aliquoted into four tubes containing 2.5 μl of the respective deoxy/dideoxy termination mixture (80 μM dATP, 80 μM dCTP, 80 μM dGTP, 80 μM dTTP, and 8 μM of the respective dideoxy nucleotide triphosphate). After a 10-min incubation at 37°C, 4 μl of stop buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) was added to each tube. Tubes were then heated to 94°C for 2 min. The reaction mixture was loaded on an 8% polyacrylamide–8 M urea gel and run for 4 h at constant wattage.

Transfection of SK-MEL-33 melanoma cells with a wild-type human B_2m gene. The procedure we used previously (8) was followed with minor modifications. Briefly, cells (1×10^7), linearized human B_2m DNA fragment (100 μg), and EcoRI-digested pSV2neo (5 μg) were suspended in 1 ml of cold PBS. An electroporation power supply (Bethesda Research Laboratories, Gaithersburg, MD) applied an electric pulse of 1 kV and 330 μF to the chamber containing cells. Cells were then recovered in RPMI 1640 medium supplemented with 10% FCS, seeded in eight 100-mm tissue culture dishes, and incubated at 37°C in 5% CO_2 for 7 d. On day 8, G418-sulfate was added to the medium at the final concentration of 2 mg/ml. The concentration of G418-sulfate was reduced to 0.4 mg/ml after 3 d of incubation and maintained at this level for an additional 10 d. Cell colonies were detached with HBSS containing trypsin–EDTA, picked up with a plastic ring, and expanded.

Results

Lack of HLA class I antigen expression by SK-MEL-33 melanoma cells. SK-MEL-33 cells did not react in a binding assay with ^{125}I-mAb W6/32 recognizing a monomorphic determinant expressed on β_2-μ–associated HLA class I heavy chains, with ^{125}I-mAb TP25.99 to a determinant expressed on β_2-μ–associated and β_2-μ–free HLA class I heavy chains, and with ^{125}I-labeled anti-β_2-μ mAb NAMB-1 (Fig. 1). No reactivity with these mAbs was detected also after incubation of cells with IFN-γ (1,000 U/ml) for 3 d before testing (Fig. 1). Furthermore, no component was immunoprecipitated from control and IFN-γ–treated ^{125}I-labeled SK-MEL-33 cells by anti-HLA Class I mAb W6/32 and TP25.99 and by anti-β_2-μ mAb NAMB-1. Neither control nor IFN-γ–treated SK-MEL-33 cells reacted with ^{125}I-labeled anti-HLA-DR, DQ, DP mAb Q5/13 (Fig. 1). SK-MEL-33 cells were sensitive to the modulating activity of IFN-γ, since IFN-γ–treated cells displayed a marked increase in the reactivity with anti-ICAM-1 mAb CL207.14 (Fig. 1).

Intracellular expression of HLA class I heavy chains and truncated β_2-μ by SK-MEL-33 melanoma cells. To determine whether the heavy chain of HLA class I antigens and β_2-μ are synthesized by SK-MEL-33 cells, an NP40 extract of [^{35}S]-methionine-labeled cells was immunoprecipitated with several anti-HLA class I mAbs, and analyzed by SDS-PAGE. No component was detected in the immunoprecipitate with anti-HLA class I mAb W6/32 and with anti-β_2-μ mAbs BBM-1, L368,

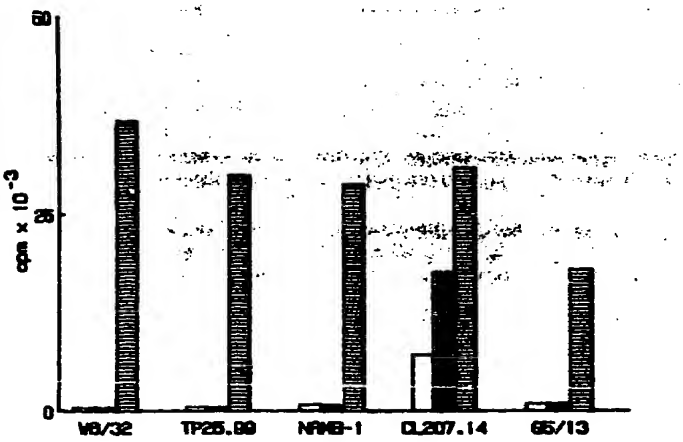

Figure 1. Lack of reactivity of control and IFN-γ–treated cultured melanoma cells SK-MEL-33 with anti-HLA class I mAbs. SK-MEL-33 cells were incubated at 37°C for 72 h with IFN-γ (final concentration 1,000 U/ml) (■). Control cells (□) were incubated under the same experimental conditions, but without cytokine. Cells were then harvested, washed twice with HBSS, and tested with ^{125}I-mAb W6/32 to a determinant expressed on β_2-μ–associated HLA class I heavy chain, ^{125}I-mAb TP25.99 to a determinant expressed on β_2-μ–associated and β_2-μ–free HLA class I heavy chain, ^{125}I-anti-β_2-μ mAb NAMB-1 and ^{125}I-anti-HLA-DR,DQ,DP mAb Q5/13 in a binding assay. ^{125}I-anti-ICAM-1 mAb CL207.14 was used to monitor the susceptibility of SK-MEL-33 cells to modulation by IFN-γ. The reactivity of radiolabeled anti-HLA mAb preparations was monitored by testing with cultured melanoma cells Colo 38 (■) in a binding assay.

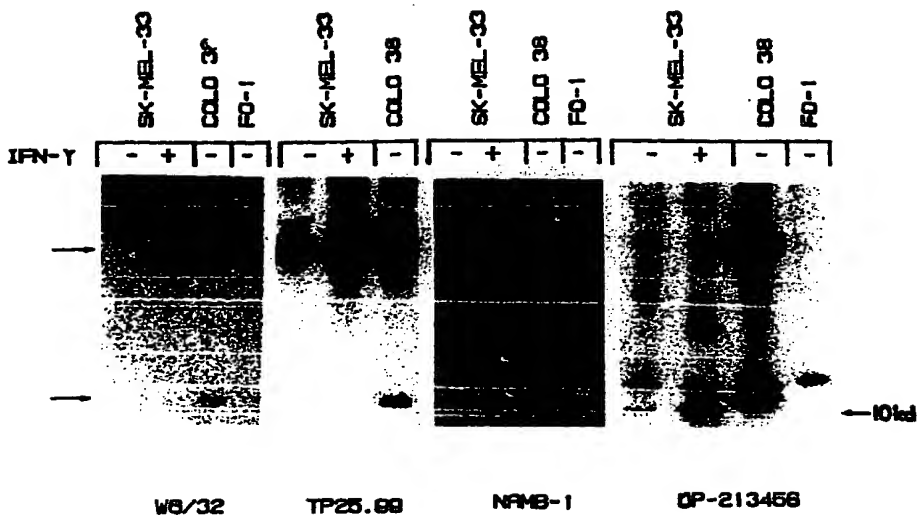

Figure 2. SDS-PAGE analysis of antigens immunoprecipitated from control and IFN-γ–treated cultured melanoma cells SK-MEL-33 by anti-HLA class I monoclonal and polyclonal xenoantibodies. After a 72-h incubation at 37°C in medium supplemented with IFN-γ (final concentration 1,000 U/ml), SK-MEL-33 cells were starved for 1 h in methionine-free medium and intrinsically radiolabeled with [^{35}S]-methionine. Control SK-MEL-33 cells were cultured and radiolabeled under the same experimental conditions but were not exposed to IFN-γ. At the end of the incubation, cells were harvested, washed three times with HBSS, and solubilized with 1% NP40. Antigens were immunoprecipitated with mAb W6/32 to a determinant expressed on β_2-μ–associated HLA class I heavy chains, with mAb TP25.99 to a determinant expressed on β_2-μ–free and β_2-μ–associated HLA class I heavy chains, with anti-β_2-μ mAb NAMB-1, and with anti-β_2-μ polyclonal antibodies isolated from rabbit anti-β_2-μ serum DP-213456. Antigens were then eluted from the immunoabsorbent and analyzed by SDS-PAGE in the presence of 2% β-mercaptoethanol. Gels were then processed for fluorography (27). Arrows indicate the position of HLA class I heavy chain and β_2-μ and of the 10-kD component precipitated from SK-MEL-33 cells. Cultured melanoma cells Colo 38, which express HLA class I antigens and cultured melanoma cells, FO-1, which synthesize HLA class I heavy chains, but do not synthesize β_2-μ (8), were used as controls to monitor the activity of the antibody preparations and the specificity of the immunoprecipitation patterns.

and NAMB-1. Representative results are shown in Fig. 2. In contrast, HLA class I heavy chains were detected in the immunoprecipitate with the mAb TP25.99 and with the xeno-antiserum R5996-4. The latter two reagents react with β_2-μ–free HLA class I heavy chains. Furthermore, a component with the apparent molecular mass of 10 kD was detected in the immunoprecipitate with anti-human β_2-μ antibodies purified from rabbit anti-human β_2-μ serum DP-213456 by affinity chromatogra-

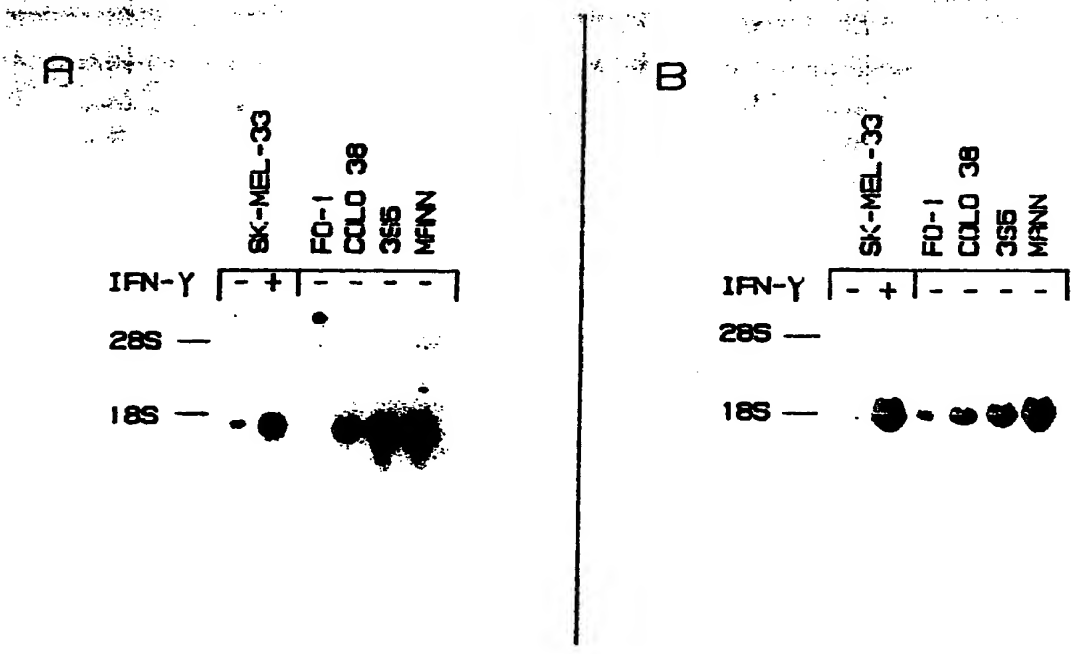

Figure 3. Northern blot analysis of HLA class I heavy chain and β_2-μ mRNA in cultured melanoma cells SK-MEL-33. SK-MEL-33 cells were incubated at 37°C for 72 h with IFN-γ (final concentration 1,000 U/ml). Control cells were incubated under the same experimental conditions, but without cytokine. At the end of the incubation, cells were harvested and total cytoplasmic RNA was extracted and hybridized with ^{32}P-labeled 0.5-kb human β_2-μ cDNA probe (*A*) and with ^{32}P-labeled 1.4-kb HLA-B7 cDNA probe (*B*). Controls analyzed for comparison purposes included RNA isolated from cultured melanoma cells FO-1, which transcribe the HLA class I heavy chain genes, but do not transcribe the B_2m genes, from cultured melanoma cells Colo 38 and 3S5 and from cultured B lymphoid cells MANN, all of which transcribe the HLA class I heavy chain and B_2m genes.

phy on β_2-μ. This component, which has a smaller size than that immunoprecipitated from [^{35}S]methionine-labeled cultured melanoma cells Colo 38, is not associated with HLA class I heavy chains. The immunoprecipitation pattern was specific, since purified rabbit anti-β_2-μ antibodies did not immunoprecipitate the 10-kD component and 12 kD β_2-μ from cultured melanoma cells FO-1, which do not synthesize β_2-μ (8). The intensity of HLA class I heavy chains immunoprecipitated by mAb TP25.99 and by rabbit antiserum R5996-4 and of the 10-kD component immunoprecipitated by rabbit anti-β_2-μ xenoantibodies was increased when the antigen source was represented by SK-MEL-33 melanoma cells incubated with IFN-γ (1,000 U/ml) for 72 h. These results indicate that SK-MEL-33 cells synthesize HLA class I heavy chains and abnormal β_2-μ; the latter had an increased electrophoretic mobility upon SDS-PAGE and lacked the ability to associate with HLA class I heavy chains.

Northern and Southern blot analysis of β_2-μ in SK-MEL-33 melanoma cells. To investigate the mechanism(s) underlying the synthesis of an abnormal β_2-μ, which does not associate with HLA class I heavy chains, the steady state level of mRNA for β_2-μ was evaluated by RNA hybridization analysis. β_2-μ mRNA was detected in SK-MEL-33 cells and had the same mobility as the β_2-μ mRNA from control cells in a 1% agarose gel (Fig. 3). The intensity of β_2-μ mRNA hybridized with radiolabeled β_2-μ cDNA probe was lower than in melanoma cells Colo 38 and 3S5; it was markedly increased when RNA was extracted from IFN-γ–treated SK-MEL-33 cells (Fig. 3). Furthermore, Northern blot analysis of RNA isolated from SK-MEL-33 cells treated with actinomycin D (final concentration 5 μg/ml) for $\leq$ 2 h detected no abnormality in the stability of β_2-μ mRNA (data not shown). Lastly, Southern blot analysis using four DNA fragments isolated from B_2m gene clone pb2m13 (8, 23) as probes detected no difference in the restric-

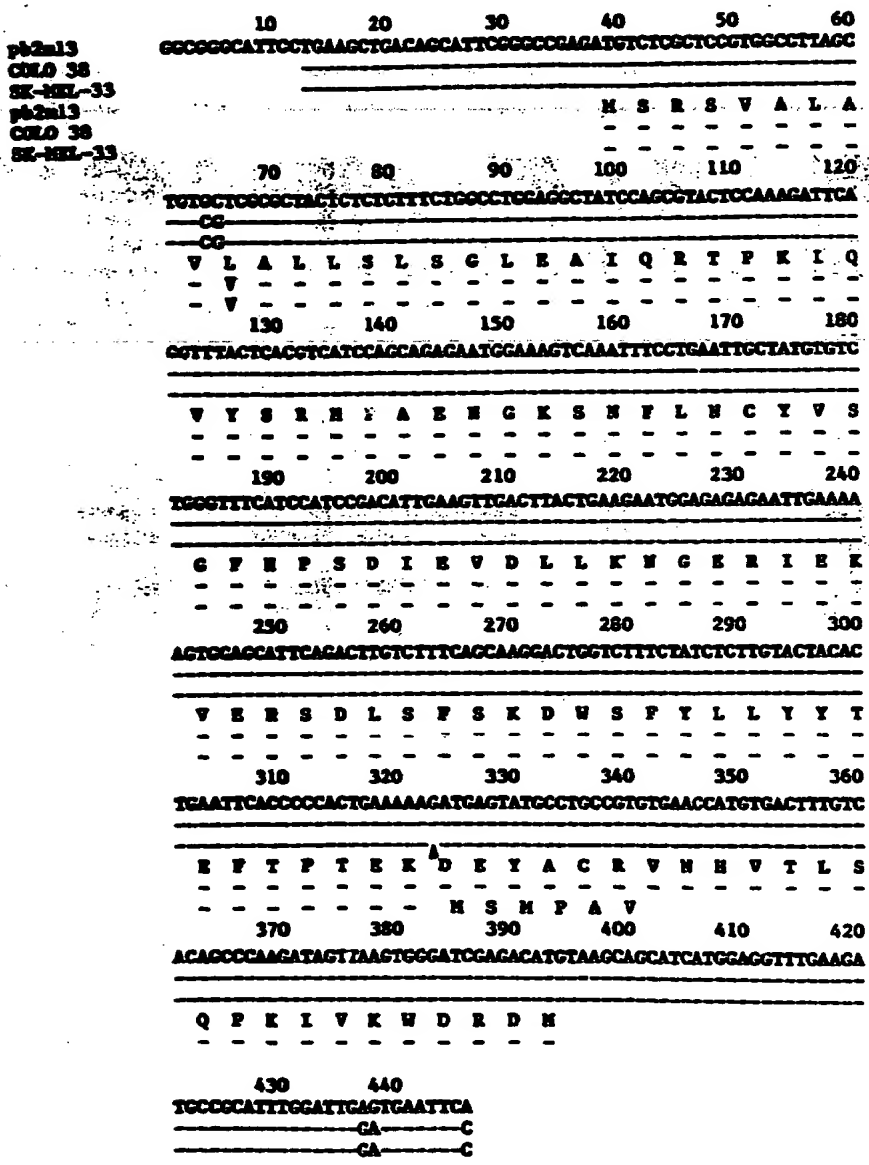

Figure 4. Comparison of nucleotide and deduced amino acid sequence of β_2-μ mRNA from SK-MEL-33 melanoma cells with that of β_2-μ mRNA from Colo 38 melanoma cells and with the published sequence of pb2m13. Dashes indicate sequence identity. The arrow indicates the base deletion detected in SK-MEL-33 cells.

tion pattern of the genomic DNA between SK-MEL-33 cells and melanoma cells Colo 38 (data not shown).

Reading frameshift mutation in β_2-μ mRNA of SK-MEL-33 melanoma cells. To determine the molecular basis for the synthesis of an abnormal β_2-μ by SK-MEL-33 cells, the β_2-μ mRNA sequence was determined. β_2-μ mRNA from Colo 38 melanoma cells, which express HLA class I antigens, was sequenced as a wild-type control. The sequences of the β_2-μ mRNA from the two melanoma cell lines displayed a 99.8% homology between themselves and of at least 98.6% with the published sequence of pb2m13 (Fig. 4). The SK-MEL-33 and Colo 38 β_2-μ mRNA sequences differ from that of pb2m13, with transition of CG to GC at positions 64 and 65, AG to GA at positions 437 and 438, and A to C at position 446. All of these nucleotide substitutions were silent except that occurring at position 65, which resulted in a switch from valine to leucine at position -11 within the leader peptide. No alteration was detected in the leader sequence and in the initiation codon of SK-MEL-33 β_2-μ mRNA. However, the base guanosine was deleted at position 323 (in codon 76) of the sequence of β_2-μ mRNA in SK-MEL-33 cells (Fig. 5). To confirm the base deletion in B_2m gene of SK-MEL-33 cells, a Southern blot analysis was performed using an oligonucleotide probe complementary to the codon 72–79 sequence of β_2-μ mRNA of SK-MEL-33 cells with the deletion of a cytosine at the region complementary to codon 76 (referred to as probe II). For this analysis an oligonucleotide complementary to the codon 1–7 of wild-type human β_2-μ (referred to as probe I) was used as a control. A temperature 5°C below the melting temperature was used for hybridization. The ^{32}P-oligonucleotide probe II hybridized to a 3.6 kb XbaI genomic DNA fragment from SK-MEL-33 cells but did not hybridize to that from Colo 38 cells. On the other hand, the ^{32}P-oligonucleotide probe I hybridized to a 4.3 kb XbaI genomic DNA fragment both from Colo 38 cells and from SK-MEL-33 cells (Fig. 6). These results corroborate the sequencing data that guanosine was deleted at position 323 (in codon 76) in β_2-μ mRNA of SK-MEL-33 cells.

The guanosine deletion causes a frameshift with a subsequent introduction of a stop codon at a position 54 bases upstream of the normal position of the stop codon in the message (Fig. 4). This results in the translation of a truncated protein that is 18 amino acids shorter than the wild-type β_2-μ protein. Furthermore, the changes from codon 76 to codon 81 result in the substitution of the corresponding six amino acids. Of great significance in terms of the overall structure of β_2-μ, is the replacement with an alanine of a cysteine that is at position 80 in the β_2-μ polypeptide.

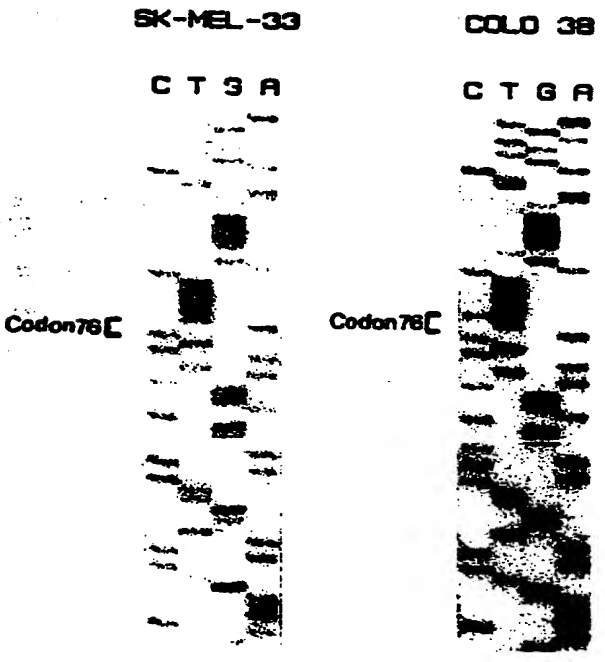

Figure 5. Sequencing gel of β_2-μ mRNA in SK-MEL-33 melanoma cells. RNA sequencing was primed with an oligonucleotide complementary to the β_2-μ mRNA at position 467–486 of 3′ untranslated region. The sequence, when read bottom to top, is complementary to the sense strand in 3′ → 5′ orientation. The sequence complementary to codon 76 is indicated. β_2-μ mRNA from cultured human melanoma cells Colo 38 was sequenced as a control.

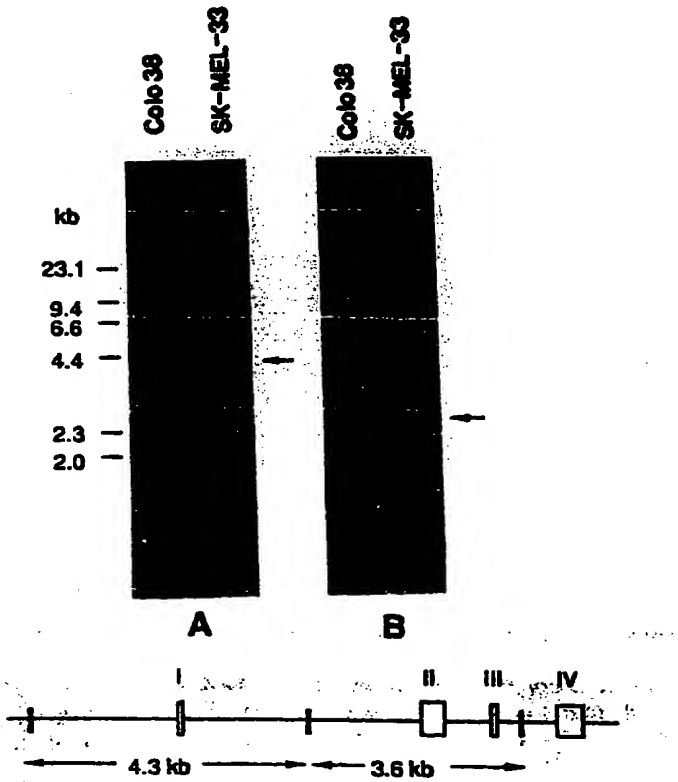

Figure 6. Southern blot analysis with oligonucleotides of B_2m gene in cultured melanoma cells SK-MEL-33. DNA (30 μg) isolated from cultured melanoma cells SK-MEL-33 (lane *2*) was digested overnight at 37°C with the restriction enzyme XbaI. Digested DNA was fractionated on a 1% agarose gel and transferred to nitrocellulose filters. Blots were hybridized with a ^{32}P-labeled oligonucleotide probe I complementary to codon 1–7 of wild-type human β_2-μ mRNA (*A*) and with a ^{32}P-labeled oligonucleotide probe II with a cytosine deletion at the region complementary to codon 76 (*B*). DNA (30 μg) from cultured melanoma cells Colo 38 (lane *1*) was run as a control. The marker lane contained λ phage DNA digested with HindIII. The bottom shows the map of the B_2m gene with restriction enzyme XbaI sites indicated by black blocks. The open blocks indicate the four exons.

Detection of a guanosine deletion in β_2-μ in melanoma tissue from which the SK-MEL-33 cell line had originated. To determine whether the base deletion detected in SK-MEL-33 cells was present in the lesion from which the cell line had originated, DNA extracted from the melanoma lesion was used for PCR amplification with primers specific for the second exon of the human gene. Approximately 200 bases of the amplified region of this gene were sequenced. The guanosine deletion in codon 76 found in the SK-MEL-33 melanoma cell line was found also in the melanoma lesion (Fig. 7).

Induction of HLA class I antigen expression on SK-MEL-33 melanoma cells by transfection with human wild-type B_2m gene. To prove that the lack of HLA class I antigen expression by SK-MEL-33 cells was caused only by the structural abnormality of β_2-μ that we have described, we determined whether transfection of SK-MEL-33 cells with a wild-type human B_2m gene could induce HLA class I antigen expression. Six clones were isolated after transfection and selection with the antibiotic G418-sulfate. Cells from five of the six clones reacted, although to a different extent, with ^{125}I-labeled anti-HLA class I mAb W6/32 and anti-β_2-μ mAb NAMB-1 (Table I). The reactivity is specific since the transfected cells continued to be unreactive with the anti-HLA-DR, DQ, DP mAb Q5/13. Furthermore, neither mAb W6/32 nor mAb NAMB-1 reacted with melanoma cells SK-MEL-33 transfected with the pSV2neo gene alone.

*Table I. Reactivity with Anti-HLA Class I mAb of SK-MEL-33 Melanoma Cells Transfected with Human Wild-Type B_2m Gene**

Cells	^{125}I-labeled mAb		
	W6/32	NAMB-1	Q5/13
	cpm × 10^{-3}		
T_2-1‡	24.2**	18.7	0.2
T_2-2	5.2	4.7	0.1
T_2-3	0.2	0.6	0.2
T_2-4	5.3	4.6	0.8
T_2-5	18.0	17.7	0.2
T_2-7	23.2	17.8	0.1
SK-MEL-33 neo§	0.2	1.4	0.7
SK-MEL-33‖	0.2	0.6	0.1
Colo 38¶	21.3	15.9	9.1

* Cells were tested with ^{125}I-mAb W6/32 to a determinant expressed on β_2-μ–associated HLA class I heavy chains and ^{125}I-anti-β_2-μ mAb NAMB-1 in a binding assay. ^{125}I-anti-HLA-DR, DQ, DP mAb Q5/13 was used as a specificity control. ‡ Clones isolated after transfection of SK-MEL-33 cells with linearized human B_2m DNA fragment and EcoRI-digested pSV2 neo and selection with the antibiotic G-418 sulfate. § Clone isolated after transfection of SK-MEL-33 cells with EcoRI-digested pSV2 neo and selection with the antibiotic G-418 sulfate. ‖ Untransfected cells. ¶ Cultured melanoma cells Colo 38 were used to monitor the reactivity of radiolabeled anti-HLA mAb preparations in a binding assay. ** cpm × 10^{-3}/1 × 10^5 cells.

Discussion

Serological and immunochemical analysis with monoclonal and polyclonal xenoantibodies has shown that cultured melanoma cells SK-MEL-33 do not express HLA class I antigens on their cell surface and do not acquire them after incubation with IFN-γ. This abnormality reflects the synthesis of a truncated β_2-μ that does not associate with HLA class I heavy chains. Since association with β_2-μ is required for the transport of HLA class I heavy chains to cell membrane (38, 39), HLA class I heavy chains that are synthesized by melanoma cells SK-MEL-33 remain in the cytoplasm and are not expressed on the cell surface. This conclusion is supported by the results of immunoprecipitation experiments performed with an extract of intrinsically radiolabeled melanoma cells SK-MEL-33 and anti-HLA class I mAb with distinct specificities. HLA class I heavy chains are immunoprecipitated by mAb TP25.99, which recognizes a determinant expressed also on β_2-μ–free HLA class I heavy chains (8), but are not immunoprecipitated by mAb W6/32, which requires the association of the HLA class I heavy chain with β_2-μ for the expression of the corresponding determinant (40).

Figure 7. Direct sequence analysis of the region surrounding codon 76 of the B_2m gene in the surgically removed melanoma lesion from which the cell line SK-MEL-33 was originated. DNA was extracted from paraffin-embedded lesion, amplified in vitro using PCR. The PCR products were ligated into plasmid vector pCR and used to transform *E. coli* INVαF. The plasmid amplified from single colonies was extracted and used in direct DNA sequencing reactions using the chain-termination technique. The sequence, when read bottom to top, is the sense strand in 5′ → 3′ orientation. The sequence shown corresponds to the stretch from codons 73 to 79.

Sequencing of β_2-μ mRNA from SK-MEL-33 detected a deletion of guanosine at position 323 in codon 76. This finding has been corroborated by the results of Southern blot analysis with an oligonucleotide complementary to codon 72–79 of SK-MEL-33 cells β_2-μ mRNA. This oligonucleotide hybridizes with genomic DNA from SK-MEL-33 cells but does not hybridize with that from Colo 38 melanoma cells without detectable abnormalities in β_2-μ. The base deletion causes a reading frameshift in β_2-μ mRNA with a subsequent introduction of a stop codon in codon 82 (54 bases upstream of the normal position of the stop codon) in the message. Therefore, the codons from 82 to 99 are not translated, causing the translation of a truncated protein that is 18 amino acids shorter than the wild-type protein. This accounts for the apparent molecular mass of 10 kD of the component immunoprecipitated by polyclonal anti-human β_2-μ xenoantibodies from intrinsically labeled SK-MEL-33 cells. Furthermore, the reading frameshift causes a missense mutation in the codons from 76 to 81 and a change in the corresponding amino acids. The latter include replacement of an alanine for cysteine at position 80, thereby disrupting an intrachain disulfide bond in β_2-μ. The loss of this disulfide bond is likely to have dramatic effects on the structure of β_2-μ in SK-MEL-33 cells, since β_2-μ has been shown to have a β-sandwich structure composed of two antiparallel β-pleated sheets joined by a disulfide linkage (41). The lack of this disulfide bond in SK-MEL-33 cells, the introduction of 6 amino acid substitutions, and the loss of 18 amino acids in the carboxy terminus of β_2-μ are likely to cause marked changes in the

conformation and structure of the polypeptide. The latter may account for the lack of association of β_2-μ with HLA class I heavy chains and the lack of reactivity with mouse anti-human β_2-μ mAb. An alternative, but not exclusive possibility for the lack of reactivity with mouse anti-human β_2-μ mAb may be the loss of the corresponding determinants located on the carboxy terminus of β_2-μ, since the most significant difference in the sequence of human and mouse β_2-μ is located at the carboxy terminus of the molecule (42). Therefore, this moiety is likely to be the most immunogenic when mice are immunized with human β_2-μ.

Karyotype analysis indicates that SK-MEL-33 cells contain two copies of chromosome 15 (unpublished results) where the B_2m gene maps (43). No rearrangement of the B_2m gene was found by Southern blot analysis of SK-MEL-33 cell DNA with fragments of wild-type B_2m gene as probes. Nevertheless, biochemical analysis demonstrated that SK-MEL-33 cells synthesize only a truncated β_2-μ polypeptide. Therefore, it is likely that a wild-type allele of β_2-μ was deleted or is nonfunctional, since melanoma cells in metastatic lesions are genetically unstable and are characterized by widespread chromosomal alterations, including genetic deletions (44). The mechanisms for loss of a functional β_2-μ allele include, (*a*) hemizygosity, with loss of a wild-type allele and duplication of the mutant B_2m gene; (*b*) deletion of the wild-type allele without duplication of the mutant allele; or (*c*) transcription of only the mutant copy of the B_2m gene due to mutations in coding or noncoding regions that are undetectable with the probes we have used. Whatever is the mechanism underlying the lack of translation of a functional β_2-μ in SK-MEL-33 cells, abnormal β_2-μ is the only defect responsible for lack of HLA class I antigen expression by SK-MEL-33 cells, since their transfection with a wild-type human B_2m gene induced their expression.

SK-MEL-33 cells are not the first example of human cells without detectable HLA class I antigen expression because of a β_2-μ abnormality. Cultured lymphoblastoid Daudi cells, which are the most extensively characterized human cells without detectable HLA class I antigen expression, transcribe the B_2m gene but do not translate β_2-μ mRNA (20, 45) because of a point mutation in the initiation codon (46). Cultured melanoma cells FO-1 do not transcribe the B_2m gene because of a deletion of the first exon, of the 5'-flanking region, and of a segment of the first intron of the B_2m gene (8). A similar defect has been identified in four mouse cell lines derived from the C48 mouse thymoma cell line R1.1 by chemical or irradiation mutagenesis and subsequent selection with anti-TL antisera and complement (47–49). Interestingly, a similar treatment of cultured human B lymphoblastoid cells has caused the mutations of genes located in the HLA class II region which control the HLA class I antigen presentation pathway (50, 51). On the other hand, no abnormalities in the structure of β_2-μ polypeptide and/or in mechanisms that regulate transcription or translation of the gene have been induced. SK-MEL-33 cells are the first example of cells with a structurally abnormal β_2-μ because of a frameshift mutation due to deletion of a single nucleotide. Furthermore, SK-MEL-33 cells represent the first example in which the same HLA class I molecular abnormality present in the cell line could be detected in the original melanoma lesion from the patient. These findings show that the lesion we have identified in the SK-MEL-33 cell line is not caused by a mutation acquired during growth in vitro, but reflects an in vivo abnormality. The lack of HLA class I antigen expression may have provided the mutant clone with a mechanism to escape from lysis by HLA class I cytotoxic T cells. The abnormality in β_2-μ is likely to reflect a somatic mutation during tumor progression, since the allospecificities HLA-A1, Aw33, B5, B14, Bw4, Bw6 were detected on patient's lymphocytes by using the complement-dependent cytotoxic assay (unpublished results). Immunohistochemical techniques could not be used to prove the lack of HLA class I antigens in the melanoma lesion, since only the formalin-fixed, paraffin-embedded tissue was available. Anti β_2-μ mAbs reacting with formalin-fixed, paraffin-embedded tissues are not available to us. Furthermore, mAbs recognizing determinants expressed only on β_2-μ associated HLA class I heavy chains stain only frozen tissue sections.

The clinical course of patient AZ was more favorable than expected, although tumor progression in patients with metastatic melanoma can be variable (52). The estimated 5-yr survival was < 20%, based on the presence of extensive regional metastases. If the lack of HLA class I antigen expression by melanoma cells played a role in the clinical course of the disease in this patient, two contrasting scenarios may be envisioned. Loss of HLA class I antigen expression could have selected for a clone of AZ melanoma cells that escaped immune recognition by HLA class I–restricted cytotoxic T lymphocytes. If so, surgery removed all melanoma cells lacking HLA class I antigens. Alternatively, lack of HLA class I antigen expression could have enhanced the susceptibility of AZ melanoma cells to NK cell–mediated lysis, thus facilitating the clearance of residual melanoma cells after the surgical excision of nodal metastases. We favor the latter possibility, since in preliminary experiments we have found a reduction in susceptibility to NK cell–mediated lysis of melanoma cells SK-MEL-33 associated with the induction of HLA class I antigens by transfection with a wild-type human B_2m gene.

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